Conditions for the Establishment of Dispersed Cell Culture of Intracranial Tumors

AUTHOR(S):

TAKEUCHI, JUJI

CITATION:

TAKEUCHI, JUJI. Conditions for the Establishment of Dispersed Cell Culture of Intracranial Tumors. 日本外科宝函 1971, 40(2): 123-145

ISSUE DATE:

1971-11-01

URL:

http://hdl.handle.net/2433/207933

Arch. Jap. Chir. 40 (2), 123~145, Nov. 1971

原　　著

Conditions for the Establishment of Dispersed Cell Culture of Intracranial Tumors

by

JUJI TAKEUCHI

Department of Neurosurgery, Kyoto University Medical School,
(Director: Prof. HAJIME HANDA)

Received for Publication : Aug. 17, 1971

The first indication of the viability of glioma tissue *in vitro* were made by FISHER (8). Then, KREDEL (13, 14), BUCKLEY (2) and BUCKLEY and EISENHARDT (3) in CUSHING's laboratory reported some success in culture of meningioma, glioblastoma, astrocytoma and acoustic neurinoma. These beginnings were soon vindicated by the value of the contributions to the biology of gliomas *in vitro* by RUSSELL and BLAND (31, 32). Their objectives were the studies of the forms of the individual migrating cells and also of the stability of the different types of cells with spongioblastomas, astrocytomas, oligodendroglioma and medulloblastomas.

Before and during the war years, further contributions were made by PINKUS (27, 28), and COX and CRANAGE (5) on gliomas *in vitro* and that of MURRAY (21-23) on the cells of the peripheral nervous system.

The post-war accleration of the research on tissue culture was considerable by the introduction of new facilities like the phase-contrast microscope, antibiotics and the roller-tube technique. However, all these works were made mainly by a tissue explant method. Tissue explant method fits for the qualitative investigation of tumor cells, but insufficient for a quantitative analysis. For the quantitative study, the following prerequisites must be satisfied; 1) all cells are equally grown on the glass surface, 2) numbers of cells in culture are enough to be analyzed quantitatively, and 3) growth media are constant in their compositions. For these purposes, cells must be cultured dispersedly. Moreover, if glioma cells are used, there is still a problem of identification of glial and glioma cells from mesenchymal cells.

Therefore, the present study was undertaken to establish an ideal method for dispersed culture of glial and glioma cells.

Part 1 is concerned with the conditions of dispersed cell culture of intracranial tumors, Part 2 with the method of purification of S-100 protein, and Part 3 with an immunofluorescent study of glial and glioma cells with S-100 protein.

Part 1 Dispersed Cell Culture of Intracranial Tumors

An establishment of the most adequate conditions for the successful culture of glial cells was necessary as an experimental model for investigation of their biology and biochemistry. A critical analysis of the following conditions necessary for culturing brain and brain tumor cells in closed stationary bottles was studied.

1. Dispersing the cells from tumor or brain tissues.
2. Concentration of bovine serum added to the synthetic media.
3. Significance of fetal calf brain extracts as a nutrient.
4. Identification of glial cells *in vitro*.

MATERIALS AND METHODS

Dispersion of Cells: Small pieces of a fresh meningioma taken at operation were minced with a scissor into several small fragments. These small fragments were washed several times in DULBECCO's PBS (7) containing 0.02 per cent EDTA (ethylenediamine tetraacetate) until the supernatant fluid became clear. Trypsinization was performed for 30 minutes with agitation of different concentration of trypsin (Difco 1 : 250) in Ca and Mg free PBS containing 0.02 per cent EDTA. Havested tumor cells were centrifuged, washed several times with PBS, and resuspended in a growth medium consisting of YLE with 20 per cent bovine serum. Number of harvested cells was counted with hemocytometer. They were then resuspended in growth medium, the concentration of cells being adjusted to be 4–6 x 10^5/ml, and placed stationarily in an incubator at 37 C.

Bovine Serum: Bovine serum was extracted from blood taken at Kyoto Slaughter House. Plating number and growth of cells, were observed daily with an inverted microscope, and examined in 5, 10, 20 and 30 per cent bovine serum in YLE, respectively.

Fetal Calf Brain Extracts: Fresh fatal calf brain of about 200 th day of gestation was homogenized in Virtis homogenizer with an equal volume of PBS. The homogenate was centrifuged at 6,000 rpm for 30 minutes and then at 60,000 x g for 60 minutes. The supernate was desinfected with Seitz filter, and stored in a cold room at 4 C. Fine filamentous sediments, formed spontaneously several days after filtration, was pipetted out. Effects of fetal calf brain extracts on cell growth in vitro were examined in newborn mouse brain cell cultures. The growth of cells was indicated by formation of cytoplasmic processes and monolayer cell sheet.

Cajal's Staining Method for Astrocytes:

1. Cells were fixed for 7 days in CAJAL's formalin-ammonium bromide mixture.
2. Refixed for 4 hours in concentrated formalin-ammonium bromide mixture.
3. Rinsed in warm pyridine-mercuric ammonium.
4. Placed in neutral 5% formalin for 10 minutes.
5. Impregnated in 1% gold chloride.
6. Placed in 5% sodium thiosulfate for 5 minutes.

7. Rinsed in distilled water.
8. Results-astrocytes would be stained dark brown.

Weigert's Staining Method for Glia Fibres:

1. Fixed for 7 days in WEIGERT's fluor chromate-cupric acetate mixture.
2. Placed in 0.3% potassium permanganate for 10 minutes.
3. Placed for 2 hours in chromogen-formic acid-sodium sulfite mixture.
4. Stained in methyl violet solution.
5. Placed for 30 seconds in iodine-potassium iodide solution.
6. Differentiated in aniline-xylene.
7. Washed in xylene.
8. Results-glia fibres would be stained purple-blue.

Alzheimer-Mallory's Staining Method for Oligodendrocytes and Astrocytes:

1. Fixed for 7 days in WEIGERT's fluor chromate-cupric acetate mixture.
2. Rinsed in dilute acetic acid for 2 minutes.
3. Stained in MALLORY's phospomolybdic hematoxylin overnight.
4. Results-oligodendrocytes and astrocytes would be stained red, and glia fibres blue.

Van Gieson's Staining Method for Connective Tissue Fibres:

1. Stained with WEIGERT's iron hematoxylin.
2. Stained in VAN GIESON's solution for 5 minutes.
3. Rinsed in distilled water and differentiated in alcohol.
4. Results-connective tissue would be stained red and background yellow.

RESULTS

1. As shown in Table 1, dispersion of tumor cells was very slight in treatment with 0.003 per cent trypsin and incomplete even with 0.3 per cent tryprin for 30 minutes. No cell destruction and subsequent cell growth in a satisfactory way

Table 1

trypsin (%)	results following trypsinization	
	cell appearance	grade of digestion
0.3	cell margine was smooth in outline without degenerative change	incomplete and several tissue clumps were seen
0.03	〃	incomplete and many tissues were left undigested
0.003	———	quite few cells were dispersed
0.1 (repeatedly)	smooth cell margine without degenerative change	nearly complete

Human meningioma cells were dispersed. Procedure of trypsinization was described in materials and methods.

indicated that the treatment with 0.3 per cent trypsin for 30 minutes seemed to be not so cytotoxic to human meningioma cells. Since the dispersion of cells was still incomplete with 0.3 per cent trypsin, refreshing of trypsin solution until the complete harvest of tumor cells from tumor tissue was considered. Since repeated treatment with 0.3 per cent trypsin might be cytotoxic to tumor cells, 0.1 per cent trypsin was refreshed several times at 15 minutes intervals until connective tissue fibers alone remained. Neary all tumor cells were harvested from tissue fragment of meningioma, as shown in Fig. 1.

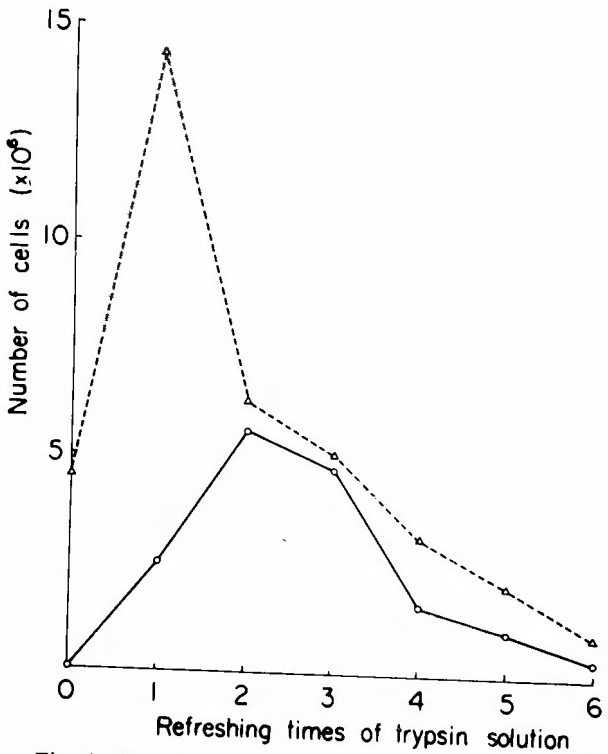


Fig. 1. Human meningioma was treated repeatedly with 0.1% trypsin and 0.02% EDTA in PBS. Number of dispersed tumor cells (solid line) and erythrocytes (broken line) were counted in each change of trypsin solution.

Table 2

	concentration of bovine serum			
	5%	10%	20%	30%
No of cells attached	50, 41, 44, 33, 44,	41, 40, 44, 44, 41,	40, 53, 48, 42, 51,	35, 41, 38, 49, 49,
mean	42.6	42.0	46.8	42.2

Human meningioma cells were used. Similar results were found in human glioblastoma cells.

2. Cell plating in 4 different growth media was almost equal in number, as shown in Table 2. However, formation of cytoplasmic processes and the subsequent increase of cell number was different in 4 growth media.

Five per cent bovine serum in the growth medium was inappropriate, indicated by no subsequent increase in numbers of survived cells in vitro (Fig. 2.). Thirty per cent bovine serum in growth medium was suitable for cell attachment to glass surface, but viability seemed not so good. On the other hand, 20-10 per cent bovine sera in the growth medium proved excellent, as was indicated by nicely extended

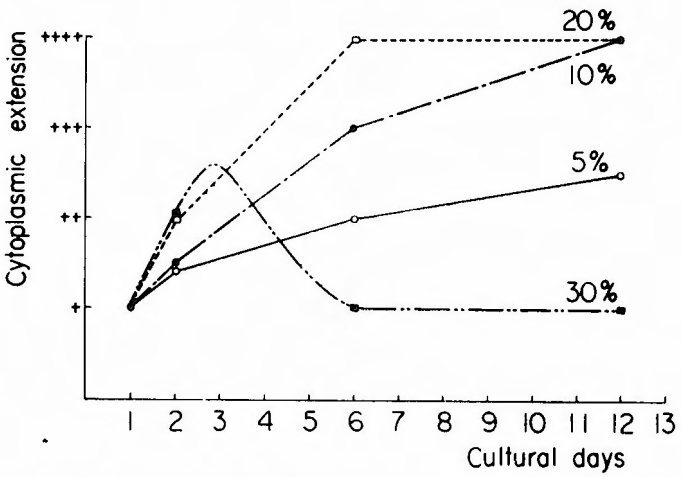


Fig. 2. Succesive formation of cytoplasmic processes in growth media containing different concentration of bovine serum is demonstrated. Cells adhered to glass surface were round, showing no cytoplasmic extension, +; a few cells with cytoplasmic extension was scatteredly observed, ++; monosheet of cells with cell processes were observed on some part of glass surface, +++; monosheet of cells with well developed cytoplasmic processes were observed on all of glass surface, ++++. Serum concentration was 5%, ∘—∘; 10%, •—·—•; 20%, ∘····∘; and 30%, ■ —··— ■.

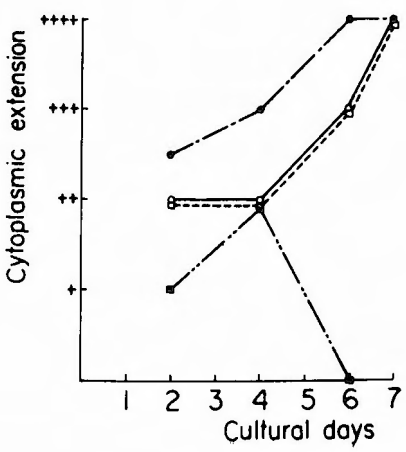


Fig. 3. Influence of 5, 10, and 15% fetal calf brain extracts (FCBE) on formation of cytoplasmic processes is shown. Signature, +, ++, +++ and ++++ means the same as in Fig. 2. Fifteen % bovine serum (BS) in Eagle MEM, ∘····∘; 15% BS and 5% FCBE in Eagle MEM, ∘—∘; 10% BS and 10% FCBE in Eagle MEM, •—·—•; and 5% BS and 15% FCBE in Eagle MEM, ■ —··— ■.

cytoplasmic processes and good monosheet of tumor cells.

3. As shown in Fig. 3, the growth medium containing 5 per cent fetal calf brain extracts proved most effective for formation of cytoplasmic extensions. This demonstrated that fetal calf brain water extracts were effective for cell attachment to the glass surface and for high plating efficiency (Table 3). The doubling time of cells, however, was not shortened.

4. Newborn mouse brain cells and human glioblastoma cells were stained with CAJAL's method (Figs. 4 and 5). Membranous glia cells which seemed very flat and thin in appearance, were very hard to be impregnated with gold. On the other hand, voluminous glial cells were usually stained brown or brownish black. The stain of glial cells *in vitro* seemed to depend on the thickness of cells rather than the glial cell types. Thread-like slender glial processes were hardly stained, whereas stout glial processes were stained light brown. Non-glial cells were usually not stained, except voluminous arachnoid cells which were stained dark brown.

Table 3

BS	15%	20%	15%
FCBE			5%
Eagle MEM	85%	80%	80%
No of cells attached	5, 4,	5, 6,	2, 6,
	1, 4,	4, 4,	6, 6,
mean	3.8	4.8	5.0

BS = bovine serum, FCBE = fetal calf brain extracts. Cells on glass surface were harvested by trypsin solution and their number was counted in hemocytometer per unit volume.

5. Nuclei of glial cells were stained pale blue by WEIGERT's staining method. Cytoplasms of glial cells were usually not stained, while voluminous portions of cytoplasms were stained faintly blue. Arachnoid cells were also stained pale blue. Some thickness of cytoplasms was necessary for staining, and no remarkable difference was observed in staining between glial and mesenchymal cells.

6. While flat, thin membranous glial cells were stained pale blue, voluminous glial cells were stained blue with ALZHEIMER-MALLAORY's staining method. Non-glial cells were stained pale blue also. Thus, no remarkable difference was observed in staining between glial and mesenchymal cells.

7. While newborn mouse heart cells were stained pink, glial cells and human glioblastoma cells were stained yellow brown (Fig. 6) with VAN GIESON's staining method. Difference in color of staining was not so definite, because some glial cells were stained as light pink or pinkish brown as the heart cells were.

Fig. 4. Six arachnoid cells, 3 membranous glial cells, and a bipolar glia cell taken from a newborn mouse brain and grown *in vitro* are shown. The arachnoid and bipolar glia cells are darkly stained, whereas the membranous glial cells are stained faintly. CAJAL's stain.

Fig. 5. Three piloid astrocytes stained moderately are found on cellular sheets of membranous glial cells. CAJAL's stain.

Fig. 6. Human glioblastoma cells were stained yellow brown, and their nuclei were stained pinkish brown. However their stainig was considerably weak as compared with that of mesenchymal cells. VAN GIESON's stain.

DISCUSSION

1. For the study of dispersion of cells, meningioma was selected, because it was fibrous and firm, and seemed hard to be dispersed with trypsin. Glioma cells also were well dispersed with the present method, exhibiting no cell death. Our digestion procedure was different in tryprinization from the method of NAKAZAWA (24), HORITA and OHYAMA (11), SATO et al (33) and MIYAKE et al (18), all of which treated tissue fragments with trypsin without refreshing, but similar in some points to the methods of YOUNGER (37) and RAPPAPORT (30). One of the similar points was that the trypsin solution was refreshed.

Differnce from the procedure of YOUNGER and RAPPAPORT were firstly concentration of trypsin and application of EDTA. As mentioned, 0.1 per cent trypsin solution containing 0.02 per cent EDTA in Ca and Mg free PBS was used. Secondly, the harvested cells were not filtered through a sterile cheese cloth before they were suspended in growth medium. Presence of cell debris occurring with digestion of tissue fragments seemed to help a cell attachment and growth on glass surface before they adapted themselves to a new environment.

2. The most appropriate concentration of bovine serum in Eagle MEM was same as in YLE (Yeast extracts + Lactalbumin hydrolysates + Earle's balanced salt solution).

Five per cent bovine serum might be inadequate for growth of cells. Thirty per cent of bovine serum might be adequate, but more frequent medium change would be necessary, probably because of promoted high metabolic rate of cells. Based on the present study of tumor cells *in vitro*, 20 and 15 per cent bovine sera were used in primary and subsequent cultures respectively.

3. It was observed in our preliminary experiment that multiplication of human glioma and newborn mouse glial cells in the synthetic media, such as YLE, EAGLE MEM and Medium 199 (MORGAN,20) was limited as compared with that of newborn mouse liver cells or heart cells, HeLa cells, strain-L cells or VERO cells. This finding suggested that the glial cells necessitated some specific nutrition. Fetal calf brain was studied whether or not it contained a substances necessary for gratifying glial cell culture.

Fetal calf brain extracts promoted cell attachment to the glass surface, but were not effective for multiplication of cells. At high concentration, they were toxic to cells.

4. Glial and non-glial cells are ontogenetically different from each other and were discriminated on the basis of their morphological aspects, mainly by specific staining methods. *In vitro* situations, their definite discrimination and identification was often difficult in many cases, particularly after a prolonged culture. For instance, astrocytes, either of protoplasmic or fibrillary type, frequently became fibroblastic in appearance after a long period of cultivation.

Newborn mouse brain cells or human malignant glioma cells on glass surface were stained with methods of CAJAL, WEIGERT, ALZHEIMER-MALLORY and VAN GIESON.

All staining methods examined in the present study failed to differentiate

definitely glial cells from mesenchymal cells. This seemed to be due to the following factors; 1) cultured glial cells on glass surface were generally so flat and thin that resulting faintness in stain could lead us to difficulties in identification of glial cells *in vitro*, and 2) different metabolic situations which might occur *in vivo* and *in vitro* could be reflected on the glial cells in both situations.

SUMMARY

Trypsinization of tissue fragment is the first important step for establishment of dispersed cell culture. Repeated treatment with 0.1 per cent trypsin and addition of 0.02 per cent EDTA were most effective for harvesting living cells from mouse brain and brain tumors and for a constant success in their subsequent growth *in vitro*. The plating and growth of cells in bottles were greatly affected by presences of bovine serum. The present study demonstrated that 20 per cent bovine serum was most efficent for the cell plating and 10–20 per cent bovine serum for the cell growth. Fetal calf brain extracts were suggested to be a candidate for accelerating the cell growth of brain tumor *in vitro*, and to contain an essential substance for continued cell growth, which was not found in synthetic media and boine serum. Human glioma cells in Eagle MEM supplemented with 15 per cent bovine serum now have vividly growing about 140 days after the initiation of culture.

Glial and glioma cells *in vitro*, when employed the classic staining methods, were very difficult in identification in a strict sense, particularly after a prolonged culture, partly because of showing frequently fibroblast-like appearance. In Part 3, a new method with antibody against S-100 protein, one of brain specific proteins, will be described for identification of glial and glioma cells in vitro.

Part 2 A Modified Method of Purification of S-100 Protein

There have been two methods of purification of S-100 protein, one being that of MOORE (19) and the other that of GOMBOS (35) or UEMURA (34) (Fig. 7). In the present study, S-100 protein from bovine brain was prepared according to the method of MOORE modified by me.

MATERIALS AND METHODS

Preparation of Extracts : Soluble proteins from bovine brain was extracted according to the method of UEMURA (34). Fresh whole bovine brain was centrifuged for 30 minutes at 6,000 rpm, and then for

Preparation of S-100 protein

Moore	Uemura
Soluble fraction	Soluble fraction
↓	↓
Sat'$(NH_4)_2SO_4$, pH 4.2	Sat'$(NH_4)_2SO_4$, pH 4.0
↓	↓
DEAE-cellulose chromatography (parabolic)	DEAE-cellulose chromatography (two steps)
↓	↓
G-200 Sephadex chromatography	Acrylamide gel electrophoresis
↓	↓
DEAE-Sephadex chromatography	DEAE-Sephadex chromatography
↓	↓
Moore	Uemura

60 minutes at 60,000 x g after homogenized in a Virtis homogenizer. To the supernate adjusted to pH 8.6 with 1N ammonium hydroxide, solid ammonium sulfate was added at 50 per cent saturation. After 2 hours of stirring, the solution was centrifuged at 6,000 rpm for 30 minutes. The supernate was also added with solid ammonium sulfate at 100 per cent saturation, after adjusted to pH 4.0 with 1N sulphuric acid. Two hours of stirring was done and the solution was centrifuged at 6,000 rpm for 30 minutes. The precipitate thus obtained was dissolved in 5 mM Tris-phosphate buffer and dialyzed against the same buffer. After centrifugation at 6,000 rpm for 30 minutes to discard precipitated substance, the clean supernate was fractionated in DEAE-cellulose column.

Preparation of DEAE-cellulose Columns: DEAE-cellulose (Brown Co.) was treated with 1N NaOH and 1N HCl for activation, followed by washing with water stored in water at 4°C. Packing of columns was performed just as described by PETERSON and SOBER (26). Columns were then equilibrated with 5 mM Tris-phosphate buffer, pH 7.2 before operation.

Elution of DEAE-cellulose Columns: The soluble brain proteins obtained above were applied to the column and allowed to soak in gravity. After all of the protein solution had entered, the column was eluted with following buffers at a flow rale of 8-10 ml per hr per cm^2.

1) 5 mM Tris-phosphate buffer, pH 7.2.
2) 0.2 M NaCl in 15 mM sodium phosphate buffer, pH 6.6.
3) 0.4 M NaCl in 20 mM sodium phosphate buffer, pH 6.6.
4) 0.6 M NaCl in 25 mM sodium phosphate buffer, pH 6.4.
5) 2.0 M NaCl in 50 mM sodium phosphate buffer, pH 6.2.

Absorbancy at 260 mμ and 280 mμ was measured in each fraction on Shimadzu UV Spectrophotometer. The fractionated protein was stored in −20°C deep freezer after dialyzed against water and lyophylized.

Preparation of Sephadex G-100 Columns: Sephadex G-100 (Pharmacia) was suspended in 0.15 M sodium phosphate buffer, pH 7.1 for swelling. The columns were packed by pouring the swelled gel to a depth of 180 cm in 1.0 cm inside diameter glass tubes with cotton bottoms. On the top of the column bed, a piece of filter paper was laid not to disturb the column bed. They were then washed with 3 to 4 column volumes of the buffer by simple gravity flow at a rate of about 9 ml per hr under 100 to 200 mmH_2O pressure. This buffer solution was also used for elution of columns.

Elution of Sephadex G-100 Columns: Lyophylized sample was dissolved in 1 ml of the buffer described above, and loaded on the column. The column was operated by simple gravity flow at a rate of 9 ml per hour with the same buffer. Absorbancy at 280 mμ and 260 mμ was also measured in each fraction. The fractionated protein was lyophylized after dialyzed against water.

Preparation of DEAE-Sephadex A-50 Columns: DEAE-Sephadex (Pharmacia) was suspended in 0.2 M NaCl in 0.1 M sodium phosphate buffer, pH 7.1 over 24 hours for swelling. The columns were packed by pouring the swelled gel to glass tubes with cotton bottoms. They were washed with about 10 column volumes of the same buffer.

Elution of DEAE-Sephadex A-50 columns: The protein fractionated on Sephadex

G-100 column chromatography and lyophylized was dissolved in the buffer of equilibration of the column and loaded on the column. After eluted with 2 column volumes of the same buffer, the column was operated with linear gradient between 0.2 M to 0.7 M NaCl in 0.1 M sodium phosphate buffer, pH 7.1. Absorbancy at 280 mμ and 260 mμ was also measured.

Protein Assay: Protein was measured by method of LAWRY et al (15), using crystalline bovine serum albumin (Armour Pharma. Co.) as control. The protein was also assayed from the optical density reading (36);

$$\text{Prmg/ml} = 1.55 \times \text{OD } 280 - 0.76 \times \text{OD } 260$$

The protein estimated by both methods was neary the same. But as the purification of S-100 protein proceeded, the former method gave consistently higher recoveries.

Molecular Weight of S-100 Protein: Molecular weight of S-100 protein was estimated on Sephadex G-100 column chromatography. Preparation and elution of Sephadex G-100 column was the same as described above. Crystalline bovine albumin, Ribonuclease A (Sigma) and Cytochrome C, type 3 (Sigma) were used as control.

Polyacrylamide Gel Electrophoresis: Polyacrylamide gel electrophoresis was carried out according to the original method of ORNSTEIN (25) and DAVIS (6). The samples were run in 7.5 and 15 per cent acrylamide concentratoin for about 1 hour in 5 mm internal diameter and 8 cm long glass tubes. The pH of the separation gel was 8.3. The amount of the protein used for each gel was 150 to 200 μg. The electrophoretic

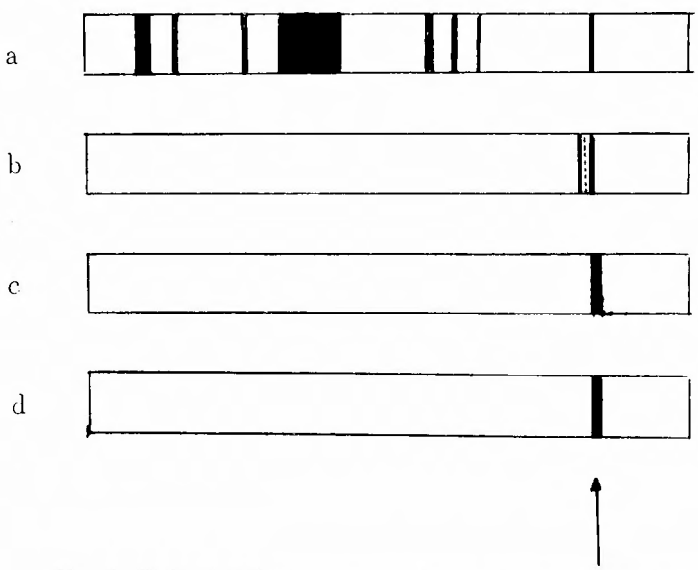


Fig 8. Stained polyacrylamide disc gels prepared and electrophoresed as described in the text to show purification of S-100 protein. Soluble proteins, a; The protein eluted in 0.6 M NaCl-25 mM Na phosphate, pH 6.4 on DEAE-cellulose column, b; purified S-100 protein on DEAE-Sephadex A-50 column, c; and purified S-100 protein on 15% polyacrylamide disc gel, d. The band of bromphenol blue is shown by the arrow.

front was shown by 0.5 ml of bromphenol blue in 100 ml of buffer solution. At the end of electrophoretic run, the gels were stained for 60 minutes in 7.5 per cent acetic acid containing 1 per cent amido black and excess dye was removed by electrophoresis at a current of 8 mA in 7.5 per cent acetic acid for about 2 to 2.5 hours. The electrophoretic separation was run at a current of 2 mA when the front line was in the concentration gel and at a current of 5 mA thereafter.

Results

On polyacrylamide gel electrophoresis (Fig. 8), S-100 protein moved with the buffer front line. On the other hand, other proteins moved far behind the buffer

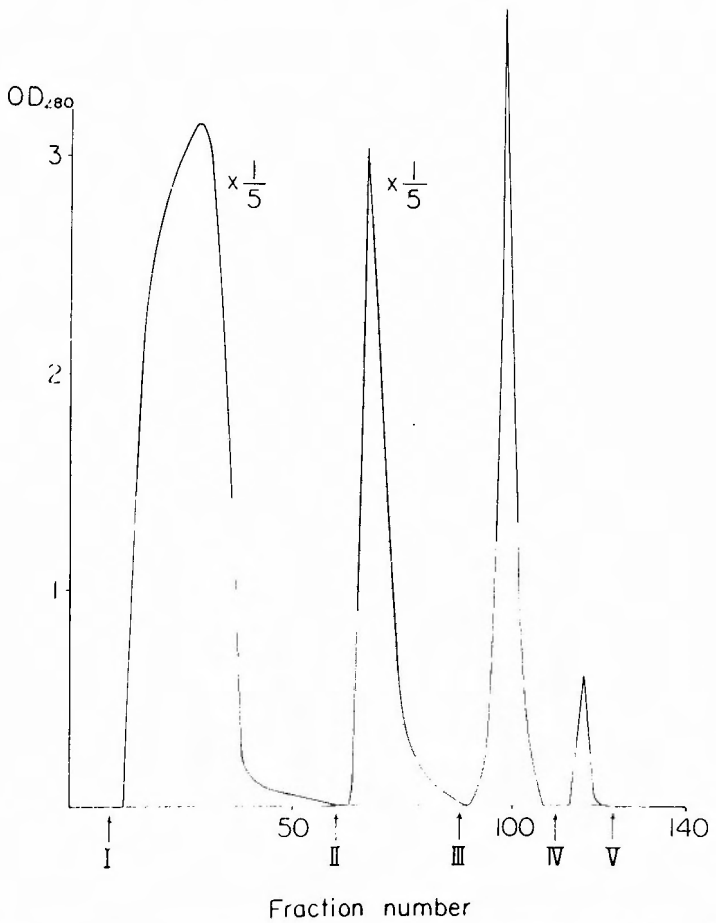


Fig. 9. Fractionation of soluble brain proteins precipitated in 100% saturated ammonium sulfate solution on DEAE-cellulose column chromatography. One hundred mg of protein in 50 ml of buffer was aplied on 1×30 cm column. Each 2.5 ml of fraction was collected. I, 5 mM Tris-phosphate, pH 7.2; II, 0.2 M NaCl-15 mM Na phosphate; III, 0.4 M NaCl-20 mM Na phosphate; IV, 0.6 M NaCl-25 mM Na phosphate; V, 2.0 M NaCl-50 mM Na phosphate.

front. These findings suggested that S-100 protein differed greatly from other proteins in molecular size and electrostatic character. These characteristics of S-100 protein could be available for its purification. Accordingly, fractionation of soluble protein with DEAE-cellulose and Sephadex G-100 column chromatography was performed, both of which being distinguished with large capacity of treatment of sample.

The soluble brain proteins precipitated in 100 per cent saturated ammonium sulfate solution, pH 4.0 were chromotographed on DEAE-cellulose column with stepwise elution (Fig. 9). Samples taken from each peak were tested for S-100 protein on polyacrylamide gel electrophoresis and S-100 protein was found only in the fraction eluted in 0.6 M NaCl-25 mM sodium phosphate buffer. This fraction was further chromatographed with Sephadex G-100 column, on the basis of molecular size difference (Fig 10). It was shown that protein thus fractionnted were separated into

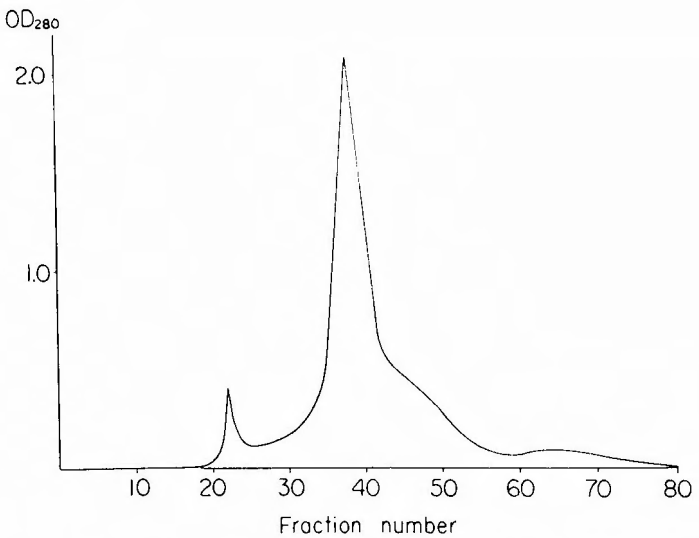


Fig. 10. Fractionation of protein solution eluted in 0.6 M NaCl-25 mM Na phosphate buffer on DEAE-cellulose column chromatography on Sephadex G-100 column chromatography.
Twenty mg of protein in 1ml of buffer was applied on column. Each 2.0 ml of fraction was collected.

2 groups, larger and smaller in molecular size. Examination with polyacrylamide gel electrophoresis showed that the first peak contained no S-100 protein, while the second peak contained only S-100 protein but no other protein. As shown in Fig. 10, the second peak seemed to be composed of at least 2 components; for the peak showed a small shoulder on its right leg. But polyacrylamide gel electrophoresis showed that the fraction contained S-100 protein only. So it was concluded that the sharp peak was composed of S-100 protein slightly contaminated with other proteins. Protein eluted in the second peak except the shoulder at the right leg was then fractionated on DEAE-Sephadex A-50 column chromatography. As shown in Fig. 11, only one

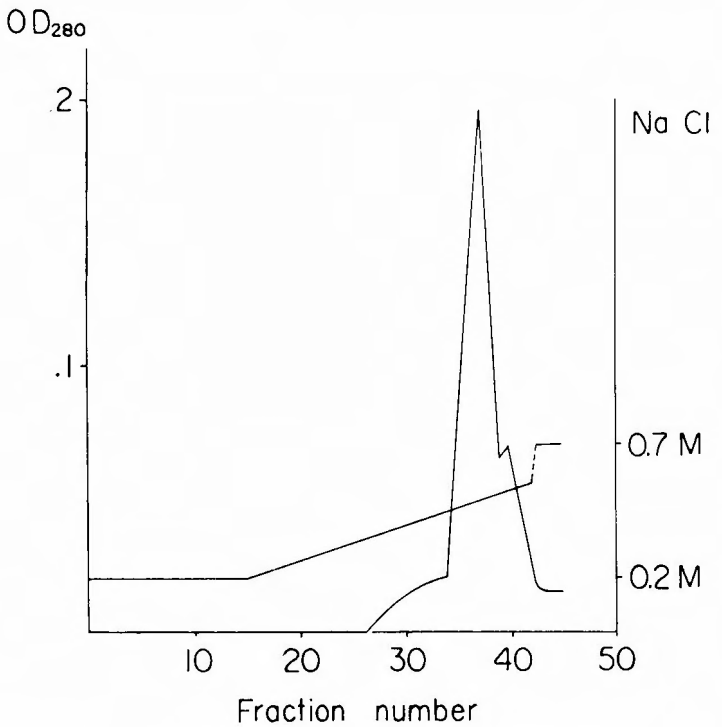


Fig. 11. DEAE-Sephadex A-50 column chromatography. One mg of protein eluted in sharp peak on Sephadex G-100 column chromatography in 1 ml of starting buffer was applied on column. Column dimensions 1×30 cm. Each 2.0 ml of fraction was collected.

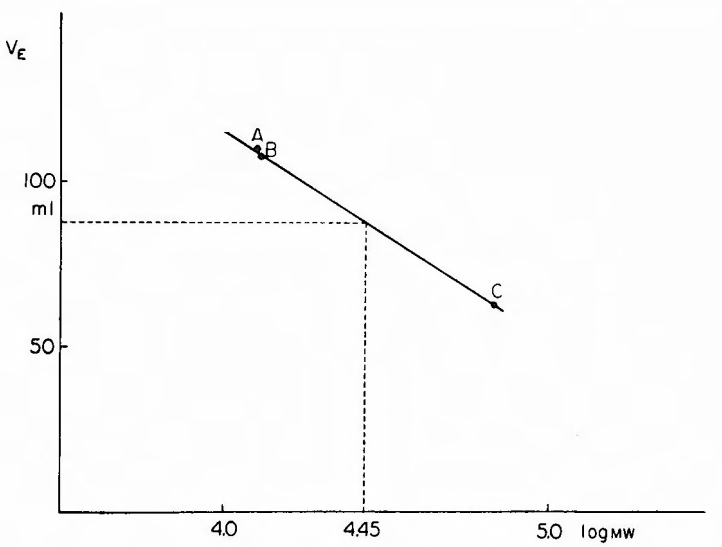


Fig. 12. Molecular weight of S-100 protein was assayed on Sephadex G-100 column chromatography. A, RNase A ; B, Cytochrome C ; C, Crystalline bovine albumin.

protein was obtained and that was S-100 protein when examined with polyacrylamide gel electrophoresis (Fig. 8).

Molecular weight of S-100 protein was assayed on Sephadex G-100 column chromatography (Fig. 12). Molecular weight thus determined was 25,700 to 30,900 (28,200) and well agreed with the Moore's result (30,000).

DISCUSSION

It was my purpose to obtain enough S-100 protein to immunize rabbit. This was attained using MOORE's method modified by me. Modification was mainly performed in the operation of DEAE-cellulose column chromatoraphy. Moore adopted parabolic gradient elution system. This system was fitted for investigation of many proteins at the same time, but rather complicated to obtain a specific protein. For this purpose, stepwise elution method was more convenient.

Several stepwise elution systems were investigated and the combination of buffers described in materials and methods was found to be most appropriate. This combination could be simplified if necessary:

1) 0.4 M NaCl-20 mM sodium phosphate, pH 6.6.
2) 0.6 M NaCl-25 mM sodium phosphate, pH 6.4.

Moore's third purification step was Sephadex G-200 column chromatography (Fig. 7). I also used this procedure because of large capacity of treatment of Sephadex gel. Considering the molecular size of S-100 protein (30,000), Sephadex G-100 gel was more appropriate rather than Sephadex G-200 gel. After chromatographed with Sephadex G-100, S-100 protein was neary pure slightly contaminated with other protein.

Final procedure of purification was DEAE-Sephadex A-50, as was the same by Moore (Table 4).

The criteria of S-100 protein purity given by MOORE (19) were: The elution in a single peak when chromatrographed on DEAE-Sephadex, Sephadex G-100 and

Table 4
Preparation of S-100 protein from 500g wet weight of bovine brain

	Moore		Author
	total protein	S-100	total protein
Soluble fraction	8.4	0.051	5.2
Sat' $(NH_4)_2SO_4$ pH 7+4.2	1.6		2.41
DEAE-cellulose chromatography	0.12 (parabolic)	0.033	0.076 (stepwise)
Sephadex chromatography	0.056 (G-200)	0.031	0.034 (G-100)
DEAE-Sephadex chromatography	0.030	0.030	0.032

hydroxylapatite columns and, above all, its imigration in a single band well ahead of other brain soluble proteins when submitted to starch gel electrophoresis in the discontinous buffer system of Poulik (29) at several pH values between 6.5 and 9.0. Procedure of purification satisfied all of the MOORE's criteria but one; the elution on hydroxylapatite column choromatography was not performed.

S-100 protein purified above had molecular weight of 25,700-30,900 (28,200), being well agreed with Moore's result, but different from VINCENDON's (35) result (15,000 -20,000).

S-100 protein thus purified was examined with 15 per cent polyacrylamide gel electrophoresis (Fig. 8). This showed purity of S-100 protein also. MCEWEN and HYDEN (17) showed that S-100 protein recovered from 7.5 per cent polyacrylamide gel electrophoresis separated into 3 bands on 15 and 20 per cent polyacrylamide gel electrophoresis. Moore (4) showed that S-100 protein could be splitted into several bands in small amount of Ca^{++}ion. My findings seemed to support MOORE's observation that S-100 is a pure protein but easily splitted into under certain conditions.

SUMMARY

S-100 protein was purified according to the method of MOORE modified by me. My procedure was simple and convenient for harvesting large amount of S-100 protein from bovine brain.

Part 3 Demonstration of S-100 Protein by an Immunofluorescent Study of Glial and Glioma Cells Cultured Dispersedly in Vitro

In Part 1, the most adequate condition for the successful culture of glial cells was studied. However, it was found that the identification of glial and glioma cells was very difficult with the classic staining methods. If we could detect S-100 protein, one of glia specific proteins, in cytoplam of cells, they would be glial cells even when their morphological appearance were similar to fibroblast like cells. Several human brain tumors and newborn mouse brain cells were investigated of existence of S-100 protein with immunfluorescent method.

MATERIALS AND METHODS

Cell Cultures: Methods of dispersion and culture of human brain tumors and newborn mouse brains were described in Part 1. Growth medium was composed of EAGLE MEM and 15 per cent bovine serum, which was inactivated at 56°C for 30 minutes, supplemented with 1 g of glucose, 100 mg of Streptomycin, 50 mg of Kanamycin, 50,000 units of Pencillin G, and 2 mg of Leucomycin per 1000 ml of medium. The growth medium was changed every 3 to 5 days.

Antiserum to S-100 Protein: Rabbit antiserum was obtained with the method as described by LEVIN and MOORE (16). The γ-globulin fraction, extracted by precipitation

with sodium sulfate according to KEKWICK's procedure (12), was purified on DEAE-cellulose column eluted with 5 mM sodium phosphate buffer, pH 8.0. This material reprsented about 88 per cent of the protein applied to the column.

Conjugation with Fluorescent Isothiocyanate (FITC): The γ-globulin fraction thus purified was conjugated with FITC (BBL) according to the method of Goldstein et al (9). Removal of fluorescent dialysable products was accomplished by passing the reaction mixtures through a column of Sephadex G-50. Conjugated globulin was frationated on DEAE-cellulose column with following buffers to obtain the fraction appropriate for staining (Fig. 13).

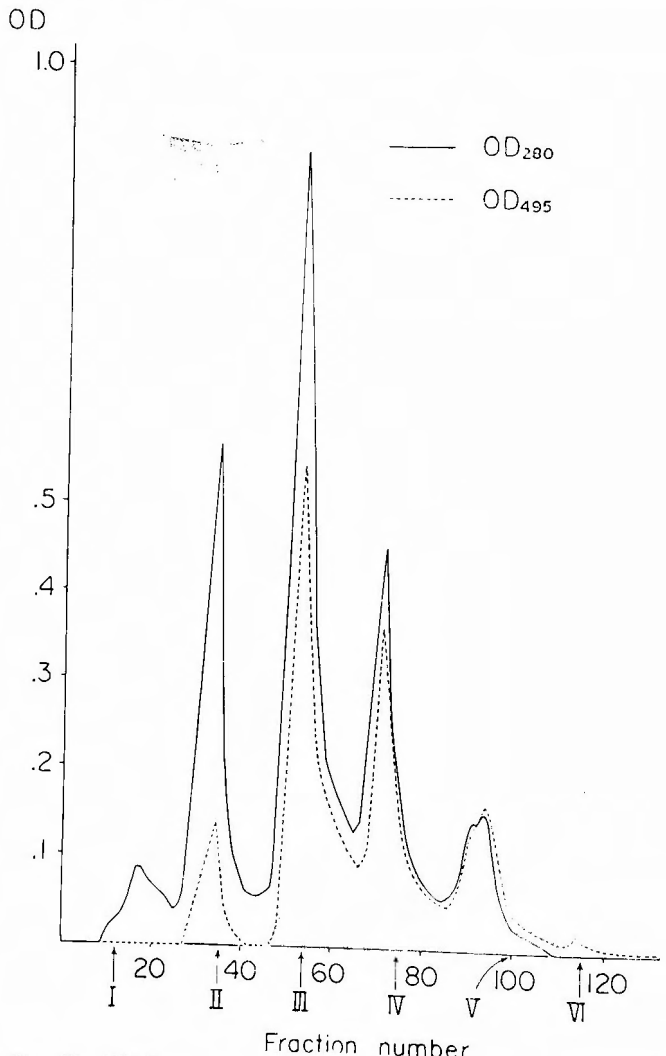


Fig. 13. FITC conjugated rabbit r-globulin was fractionated on DEAE-cellulose column chromatography. About 10 mg of protein was applied on 2×35 cm column. I, 0.01M Na phosphate, pH 7.2; II, 0.05 M NaCl-0.01 M Na phosphate; III, 0.15 M NaCl-0.01 M Na phosphate; IV, 0.25 M NaCl-0.01 M Naphosphate; V, 0.50 M NaCl-0.01 M Naphosphate; VI, 1.0M NaCl-0.01 M Na phosphate,

1) 0.01 M sodium phosphate buffer, pH 7.2.
2) 0.05 M NaCl in the same buffer.
3) 0.15 M NaCl in the same buffer.
4) 0.25 M NaCl in the same buffer.
5) 0.50 M NaCl in the same buffer.
6) 1.0 M NaCl in the same buffer.

The FITC conjugated globulin eluted in the second buffer was used.

Staining of Slides: Staining of cells cultured on slide glass was performed according to the method of HAMASHIMA and KYOGOKU(10). The buffer used throughout the staining procedure was 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2. Cultured cells were fixed in cold pure methanol for 10 minutes. The samples were then treated with FITC conjugated globulin at 37°C for 60 minutes.

Fluorescent Microscope: A Zeiss power supply powered with an Osram HBC-200 light source was used with a BG-12 exiter filter and Zeiss barrier filters. A bright field condensor was used.

RESULTS

Specific fluorescence was observed in cytoplasms of newborn mouse piloid astrocytes and of fibroblast-like astrocytes. (Fig. 14, 15), human astrocytoma cells (Fig. 16), and human neurinoma cells (Fig. 17, 18). No fluorescence was detected in nuclei of cells examined. Strong fluorescence was noted in cytoplasms around nuclei, which became gradually weak in the peripheral part of cytoplasms. When cultured cells were very flat and thin, as was observed with long cultured astrocytes, fluorescence was not strong but significant. Glial processes of cultured cells were also cleary stained.

DISCUSSION

The direct fluorescent antibody technique applied to cultured human glioma cells and newborn mouse brain cells demonstrated the specific fluorescence in their cytoplasms. No specific fluorescence was observed in nuclei of any cells examined. The strong fluorescence in cytoplasms around nuclei probably indicated that this protein was produced mainly by/near membrane structures around nuclei and transported to other parts of cytoplasms.

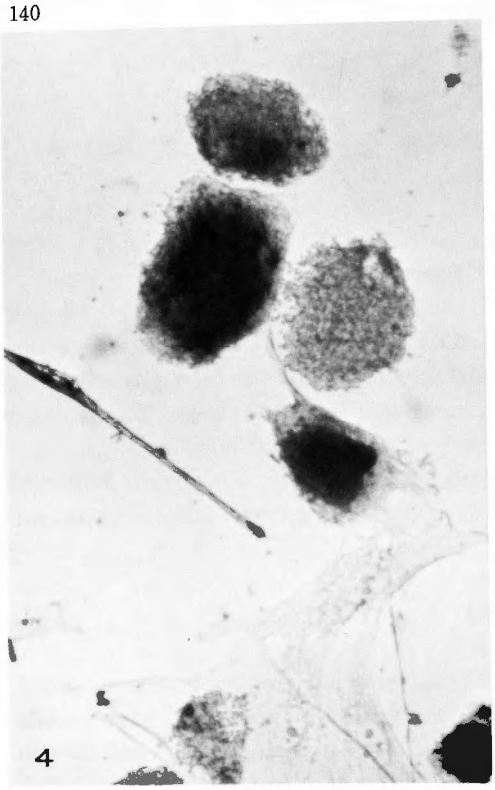
Fig. 14. Newborn mouse brain cells were stained with FITC-globulin. Strong fluorescence was detected in cytoplasms of two piloid strocytes (upper and lower) and weak fluorescence in cytoplasm of 4 fibroblast-like strocytes. Nuclei of all cells did not react.

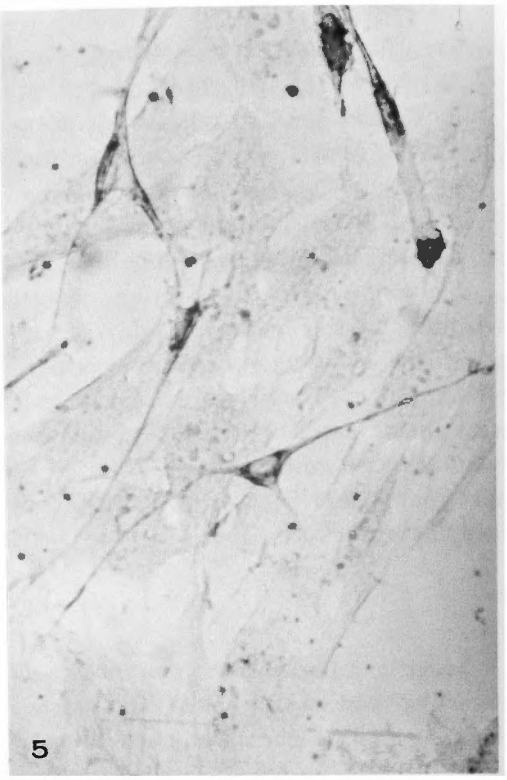
Fig. 15. Newborn mouse brain cells were stained with FITC-globulin. Cells represented are protoplasmic astrocytes except a few spindle shaped cells (spongioblasts).

Fig. 16. Human astrocytoma cells were stained with FITC-globulin. Specific fluorescence in both cytoplasms and glial processes were shown.

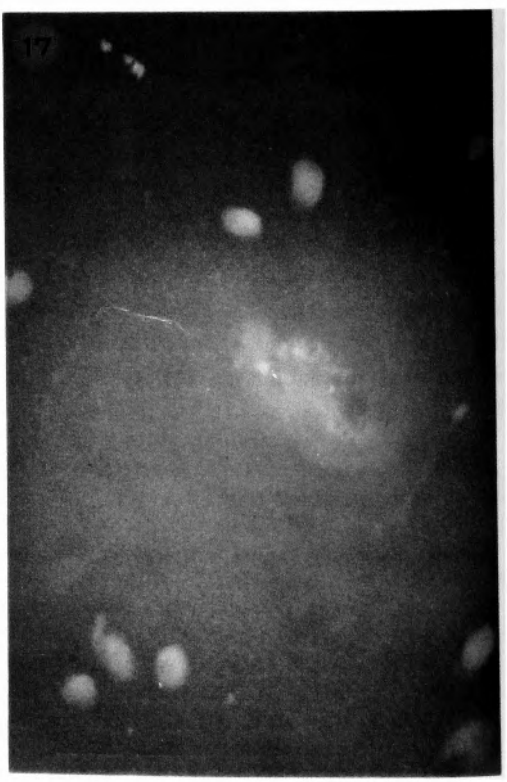
Fig. 17. Neurinoma cells of early stage of culture were shown. Specific fluorescence was detected not only round cells but also in a multinucleated giant cell (center).

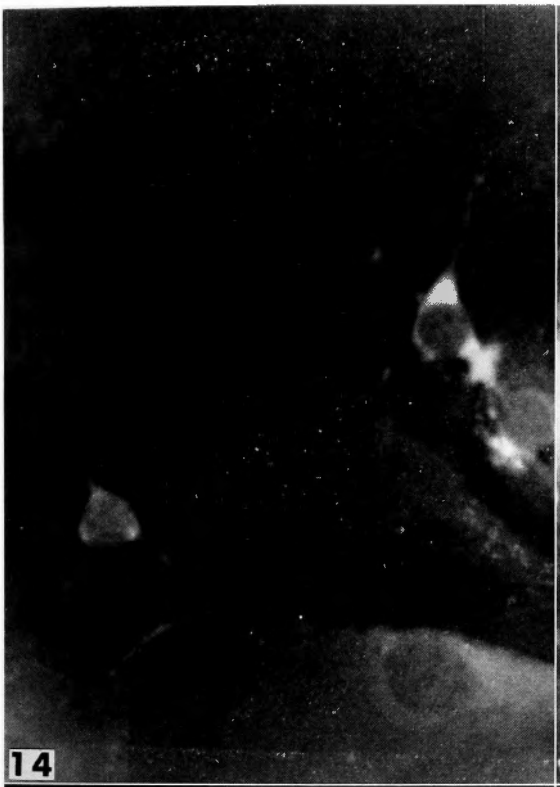
Fig. 18. Neurinoma cells of vividly growing stage of culture were shown. Specific fluorescence was detected in cytoplasms around nuclei.

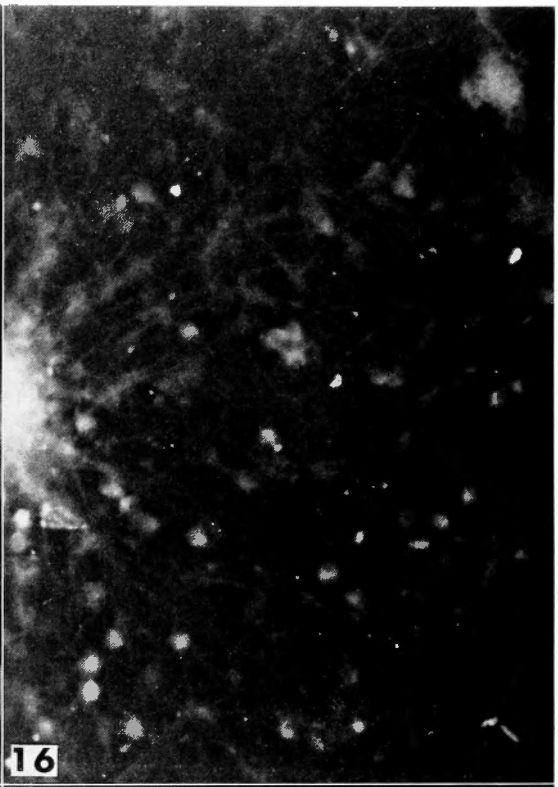

4

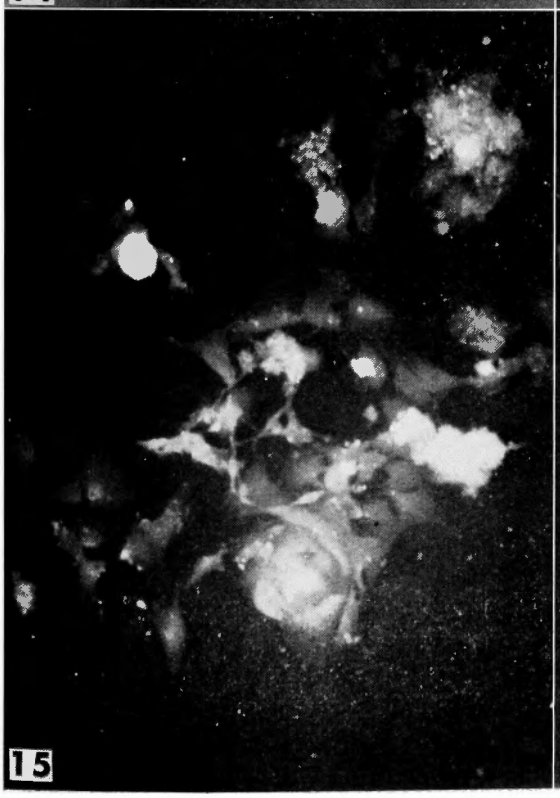

5


6


17


14


16


15


18

BENDA et al (1) demonstrated that 2 of 5 cloned rat glioma cells containing S-100 protein grew up to gliomas when implanted back to syngeneic animals. On the other hand, 2 of 3 cloned cells showing no S-100 protein grew no tumors when implanted. It was possible from these findings that cells containing no S-100 protein were not glial cells but mesenchymal cells contaminated in culture. Thus, identification of glial cells cultured *in vitro* from mesenchymal cells would be easy and reliable if S-100 protein could be detected in cytoplasms of cells.

SUMMARY

Newborn mouse brain cells, human glioma cells and human neurinoma cells culturd *in vitro* were investigated of existence of S-100 protein with direct immunofluorescent method. Cytoplasms of all of cells investigated reacted but nuclei of them did not. This method would be applied to identify glial cells from mesenchymal cells cultured *in vitro*.

CONCLUSION

For investigation of gliomas and other intracranial tumors cultured in vitro, following basic problems of cultural conditions were examined.

1. Dispersion of cells from tissue fragments.
2. Cultural media.
3. Identification of cultured cells with classic staining methods.
4. Identification of cultured cells with immunofluorescent method.

This enable us to investigate cultured glioma and intracranial tumor cells biologically and, especially, biochemically.

Acknowledgements: Part of this investigation was done in Dep. Phys. Inst. Virus Res., Kyoto Univ.. I wish to express sincere thanks to Prof. N. HIGASHI and to Dr. A. TAMURA for encouragements and helpful advice during this study. I am also indebted to Prof.H. HANDA and to Instructor E. TANI for valuable suggestions, discussions and encouragements throuought this course of this study.

REFERENCE

1) Benda, P,. Someda, K., Messer, J. and Sweet, N. H.. Morphological and immunochemical studies of rat glial tumor and clonal strains propagated in culture. J. Neurosurg., 34; 310–323, 1971.
2) Buckley, R. C.: Tissue culture studies of the glioblastoma multiforme. Am. J. Path., 5:467–472, 1929.
3) Buckley, R. C. and Eisenhardt. L.: Study of a meningioma in supravital preparations, tissue culture and paraffin sections. Am. J. Path., 5: 659–664, 1929.
4) Calissano, P., Moore,B. W. and Friesen, A.: Effect of calcium ion on S-100, a protein of the nervous system. Biochemistry, 8: 4318–4326, 1969.
5) Cox, L. B. and Cranage, M.: Studies on the tissue culture of intracnial tumors. J. Path. Bact., 45; 477–499, 1937.
6) Davis, B. J.: Disc electrophoresis 2. Method and application to human serum proteins. Ann.

NY Acad. Sci., **121**: 404–427, 1964.
7) Dulbecco, R. and Vogt, M.: Plaque formation and isolation of pure lines with poliomyelitis viruses. J. exp. Med., **99**: 167, 182, 1954.
8) Fisher, A.: Observation on the division of sarcoma cells in vitro. J. Cancer Res., **9**: 71, 1925,
9) Goldstein, G., Slizes, I. S. and Chase, M. W.: Studies on fluorescent antibody staining. 1. Non-specific fluorescence with fluorescein-coupled sheep antirabbit globulins. J. exp. Med., **114**: 89–109, 1961.
10) Hamashima, Y. and Kyogoku, H.: Immunohistology (2nd ed.) Igakushoin, 1968.
11) Horita, S. and Ohyama, A.: Fundamentals and practice of tissue culture. Nagai Book Co. 1963.
12) Kekwick, P. A.. The serum proteins in multiple myelomatosis. Biochem. J., **34**: 1428–1257, 1940.
13) Kredel, F. E.: Tissue culture of intracranial tumors, with note on meningiomas. Am. J. Path, **4**: 337–340, 1928.
14) Kredel, F. E.: Intracranial tumors in tissue culture. Arch. Surg., **18**: 2008–2018, 1929.
15) Lawry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**: 265–275, 1951.
16) Levin, L. and Moore, B. W.: Strctural relatedness of a vertebrate brains acid protein as measured immunochemically. Neurosci. Res. Progr. Bull., **3**: 18, 1965.
17) McEwen, B. S. and Hedén, H.: A study of specific brain proteins on the semi-micro scale. J. Neurochem., **13**: 823–833, 1966.
18) Miyake, S., Toyama, M., Fukuma, S., Fujita, Y., Nishioka, B., Tanaka, T. and Koike, A.: Three morphological patterns of cells produced by cultured astrocytoma-glioblastoma series. Brain and Nerve, **20**: 593, 1968.
19) Moore, B. W.. A soluble protein characteristic of the nervous system. Biochem. Biophys. Res. Comm., **19**: 739–744, 1965.
20) Morgan J. F., Morton, H. J. and Parker, R. C.: Nutrition of animal cells in tissue culture. 1. Initial studies on a synthetic medium. Proc. Soc. exp. Biol., **73**: 1–8, 1950.
21) Murray, M. R. and Stout, A. P.: Schwann cell versus fibroblast as origin of specific nerve sheath tumor; observations upon normal nerve sheath and neurilemmomas in vitro. Am. J. Path., **16**: 41–60, 1940.
22) Murray, M. R. and Stout, A. P.: Demonstration of the formation of reticulin by schwannian cells in virto. am. J. Path., **18**: 585–593, 1942.
23) Murray, M. R.: Comparative data on tissue culture of acoustic neurilemmomas and meningioma. J. Neuropath. exp. Neurol., **1**: 123, 1942.
24) Nakazawa, T.: Tissue culture of neuroglia cells. Recent Adv. Res. Nerv. Syst., **6**: 2, 1963.
25) Ornstein, L.: Disc electrophoresis 1. Background and theory. Ann. NY Acad. Sci., **121**: 321–349, 1964.
26) Peterson, E. A. and Sober, H. A.: Chromatography of proteins 1. Cellulose ion-exchange adsorbents. J. Am. Chem. Soc., **78**, 751–755, 1956.
27) Pinkus, H.: The isolation of pure strains of cells from human tumors. 1. Selections of specimens and technique. Am. J. Cancer, **26**: 521–528, 1936.
28) Pinkus, H.: The isolation of pure stains of cells from human tumors. 2. Growth characteritics of a sarcoma and two brain tumors in tissue culture. Am. J. Cancer, **29**: 25–46, 1937.
29) Poulik, M. D.: Starch gel electrophoresis in a discontinuous system of buffers. Nature, **180**: 1477–1479, 1957.
30) Rappaport, C.: Trypsinization of monkey kidney tissue: automatic method for preparation of cell suspensions. Bull. World Health Org., **14**: 147–166, 1956.
31) Russell, D. S. and Bland, J. O. W.: Study of gliomas by method of tissue culture. J. Path. Bact., **36**: 273–283, 1933.
32) Russell, D. S. and Bland, J. O. W.: Further nothes on tissue culture of gliomas with special reference to Bailey's spongioblastomas, J. Path. Bact., **39**: 375–380, 1934.
33) Sato, F., Chigasaki, F., Yamada, H., Hoshino, T., Nagai, M., Tsuchida, T. and Sano, K.;

Biochemistry and tissue culture of brain tumors. Brain and Nerve, **18**: 249–257, 1966.
34) Uemura, K. and Mandel, P. Neurochemistry (Tokyo) **7**: supple. 25, 1968.
35) Vincendon, G., Waksman, A., Uemura, K., Tardy, J. and Gombos, G.: Ultracentrifugal behavior of beef brain S-100 protein fraction. Arch. Biochem. Biophys., **120**: 233–235, 1967.
36) Warburg, O. and Christian, W.: Isolierung und Kristallisation des Gerungsferments Enolase. Biochem. Z., **310**: 384–421, 1941.
37) Youngner, J.: Monolayer tissue cultures. 1. Preparation and standardization of suspension of trypsion-dispersd monkey kidney cells. Proc. Soc. exp. Biol., **85**: 202–205. 1954.

和文抄録

頭蓋内腫瘍の培養条件

京都大学医学部脳神経外科教室（主任：半田肇教授）

武　内　重　二

頭蓋内腫瘍細胞の培養のための諸種の条件の検討を行なった．

腫瘍片よりの細胞の収集法に関しては，0.1% trypsin—0.02% EDTA 液（溶媒は Ca 及び Mg 不含の PBS）を15分毎に数回処理して，細胞をほぼ完全に収集し得た。この処理による細胞破壊は認められず，細胞の活生度も良好であった．

培養液は YLE, Eagle MEM その他の合成養液に著明な差はみられなかったが，加える牛血清の量は細胞の発育・増殖に大きな影響を及ぼした．細胞のガラス面への附着には30%濃度牛血清がよく，その後の発育・増殖には10～20%濃度が至適であった．

神経膠腫細胞の増殖は同一培養条件に於て他のL細胞，VERO 細胞，新生ネズミ心の肝細胞等にくらべ悪く，培養液中には栄養的欠陥があるのでないかと考えられた．牛胎児脳（胎生約200日）中にかかる因子を求めて，胎児脳水抽出物を作り，これを培養液中に添加した．胎児脳水抽出物は細胞のガラス面への附着，発育をうながしたが，増殖率は不変であった．

神経膠細胞・神経膠腫細胞の同定は形態学的に典型的なものは容易であったが，線維芽細胞様の形態をとるものにはその同定が困難であった．それは Cajal, Meigert, Alzheimer-Mallory, van Gieson 染色等によっても同様であった．神経に特異的である Moore の S-100 蛋白を Moore の方法を改良して変性なく容易に抽出精製して，それを用いてウサギ抗血清を作り，FITC をラベルした．これを用いての免疫螢光抗体法による神経膠，神経膠腫細胞の同定の試みは上記の染色法にくらべ信頼度が高いと思われた．